Journal of Medical Virology 49:91–94 (1996)

Nucleotide Sequences of the Hepatitis C Virus Core Region in Patients Without Anti-Core Antibody

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Second-generation assays for detection of hepatitis C virus (HCV) infection that include reactivity of antibodies to core, NS3, NS4 are used because of their high sensitivity. Among these antibodies, anti-core antibody seems to be the most sensitive. However, there are some patients without anti-core antibodies, although HCV RNA is detectable by reverse transcription-polymerase chain reaction and branched DNA assay. The mechanism for the absence of anti-core antibody on its own is unclear. We therefore determined the nucleotide and deduced amino acid sequences of the core region obtained from two anti-core antibody-negative patients with HCV RNA (genotype 1b) and compared them with those of four anti-core antibody-positive patients and a previously reported sequence. Amino acids spanning 1–47, which seemed to exist in major B cell epitopes, were found to be completely conserved among these patients. Furthermore, the predictive binding motif to HLA DR4 (a.a 81–90) was completely conserved in both of the anti-core antibody-negative patients. There were various mutations in the residual amino acids spanning 49–108, but specific mutations could not be found in anti-core antibody-negative patients.

These data indicate that the absence of anti-core antibody in two patients is not due to the presence of some formerly unknown viral variants, but due to a possible defect in the host's immune system.

KEY WORDS: hepatitis C virus, anti-core antibody, sequences of core region, mutation

INTRODUCTION

With the identification of the hepatitis C virus (HCV) and the subsequent development of the first anti-HCV antibody assay [Kuo et al., 1989], most patients with postransfusion non-A, non-B hepatitis have been diagnosed as HCV-infected [Lok et al., 1991]. Since the introduction of a second-generation anti-HCV antibody enzyme immunoassays, the sensitivity for diagnosing HCV infection has increased significantly [McHutchison et al., 1992; Jeffers et al., 1992], and the specificity has been improved by the development of confirmatory tests such as RIBA2 [Boudart et al., 1992; Follet et al., 1991; van der Poel et al., 1991]. This assay has recombinant antigens from the core region and the nonstructural (NS) regions (NS3 and NS4). Using this assay, antibodies against NS3 and the core region can be detected in most but not all HCV-infected individuals [Bresters et al., 1993].

Results of the first-generation ELISA depended on the genotypes of the HCV-infected patients [Nagayama et al., 1993]. The patients infected with HCV isolates of the genotypes 1a and 1b were significantly and more frequently positive for antibody to the C100-3 and the 5-1-1 proteins than those infected with isolates of the genotypes 2a and 2b. This difference should reflect the previously observed genetic heterogeneity in the NS4 region of these HCV genomes [Tsukiyama-Kohara et al., 1993]. Nagayama et al. [1993] also detected antibodies against the C22-3 and C33c proteins in all 155 patients studied, regardless of their HCV genotype. It seems that there are immunogenic epitopes within these proteins which are well-conserved. However, it has been found that some patients are negative for the anti-core despite the presence of a second-generation anti-HCV antibody and HCV RNA. An interesting question is whether the absence of the anti-core antibody in the patients is due to the presence of some formerly unknown vial variants or a defect in the host's immune system. We therefore determined the nucleotide and deduced the amino acid sequences of the HCV core region of anti-core-negative patients.

Accepted for publication January 2, 1996.

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PATIENTS AND METHODS

Serum Samples and HCV Antibodies

Patient 1 was a 51-year-old woman with positive EIA 2 (Abbott Laboratories, North Chicago, IL) and HCV RNA with normal alanine aminotransferase (ALT) level for 3 years. Examination by Matrix HCV (Dinabot Inc., Japan) showed that the anti-core antibody was negative, although she was positive for anti-NS3, anti-NS4(Y) and anti-NS4(E) with a cut-off index of 17.9, 8.6 and 8.8, respectively. The anti-core antibody was also found to be negative by Ortho HCV Core-Ab IRMA and CHEMO-SERO EIA JCC2 (Ortho Diagnostics Systems, Japan). The HCV genotype was 1b. The viremic level was below 0.5 Meq/ml by the bDNA assay (Quantiplex Version 1.0; Chiron Corp., CA, USA). She had no history of blood transfusions and no serological evidence of hepatitis B and autoimmune hepatitis. She had received neither immunosuppressive therapy nor interferon therapy. The HLA types were A24, A31, B54, B62, Cw1, DR4, and DR8.

Patient 2 was a 53-year-old man who had histological evidence of liver cirrhosis. He had received a blood transfusion about 30 years ago. EIA 2 and HCV RNA were positive, but both the Ortho HCV Core-Ab test and the anti-JCC2 results were found negative. He was negative for anti c22-3, but positive for anti-c33c, anti 5-1-1 and anti c100-3 in the RIBA2. The HCV genotype was 1b. The viremic level was 9.7 Meq/ml by the bDNA assay. As the control group, four patients were selected with positive EIA 2, HCV Core-Ab and HCV RNA (genotype 1b) who were histologically proven to have chronic active hepatitis.

Sequencing of Core Region

RNA was extracted from 50 μl of serum by treatment with 500 μl of guanidinium thiocyanate buffer (4.0 mol/L of guanidinium thiocyanate, 0.1 mmol/L Tris-HCl [pH 7.5], 1% 2-mercaptoethanol, and 0.5% sodium lauryl sarcosinate), 50 μl of sodium sulfate (pH 5.2), 500 μl of phenol (pH 7.2), and chloroform/isoamylalcohol compound (24:1), and was then precipitated with isopropanol. Complementary DNA (cDNA) was synthesized with molony murine leukemia virus reverse transcriptase (GIBCO BRL, Gaithersburg, MD), RNase inhibitor (Promega, Madison, WI) and random primers (Takara Biomedicals, Kyoto, Japan).

HCV-cDNA was amplified with the polymerase chain reaction (first-round PCR) using the procedures listed as follows: 2.5 μl of the cDNA reaction mixture was mixed with 2.5 μl of 10 × PCR buffer containing $MgCl_2$, 0.5 μl of 10 mM dNTP mix, 5 pmol each of the sense- and antisense-strand primers, and 1 unit of Taq polymerase (Perkin Elmer Cetus, Norwalk, CT) to a final volume of 25 μl. In general, each reaction cycle was carried out at 94°C for 1 minute, 55°C for 1.5 minutes, and 72°C for 1.5 minutes. A total of 40 reaction cycles were carried out. The sense primer used was 5′TCACTCCCCTGTGAGGAACT3′ and the anti-sense primer used was 5′TGACATGGTATACCCCGGACGCGTT3′. Using these two primers, the HCV sequence from nucleotides 36 to 950 was isolated [Takamizawa et al., 1991]. This sequence contains the entire core gene sequence plus the 5′-untranslated and a short stretch of the E1 gene sequence. Ten microliters of PCR products were applied to 4% agarose gel (NuSieve GTG., FMC Corp., Rockland, ME, USA) and electrophoresis was carried out. The gels were stained with ethidium bromide and examined for the presence of bands of the expected size under ultraviolet fluorescence. The bands were excised and the cDNA fragments were extracted with SUPREC™-01 (Takara Biomedicals). The cDNA was then diluted with 50 μl of sterile H_2O. For asymmetric PCR, 100 μl of reaction mixture containing 2 μl of diluted cDNA, 10 μl 10 × PCR buffer containing $MgCl_2$, 1.6 μl of 10 mM dNTP mix, 1 pmol of one primer, 100 pmol of the other primer, and 4 units of Taq polymerase were prepared, and 40 cycles of 94°C for 20 seconds, 50°C for 20 seconds, and 72°C for 30 seconds were carried out. The sense primer used was 5′TGTTGGGTCGCGAAAGGCCT3′ and the antisense primer used was the same as the primer of the first-round PCR. Asymmetric PCR products were treated with SUPREC™-02 (Takara Biomedicals) to remove the residual primers and dNTP mix. Single-strand cDNA was recovered from the aqueous phase by precipitation with 500 μl of 80% ice-cold ethanol containing 20 μg of glycogen (Boehringer-Mannheim, Mannheim, Germany) as a carrier. The determination of the sequence was carried out by the dideoxy chain termination method using a Sequenase Version 2.0 Labeled dCTP Kit (United States Biochemical Corp., Cleveland, OH). The sequence primer was the same as the sense primer used for the asymmetric PCR. To eliminate a possibility of Taq incorporation errors, two independent amplification products were sequenced for each sample.

HCV Genotype

HCV genotypes were determined by two-stage PCR using mixed primers which were derived from the putative core region according to the method of Okamoto et al. [1992]. The terminology of the genotypes was that described by Simmonds et al. [1993].

RESULTS

For the current study, six serum samples from two anti-core antibody-negative and four anti-core antibody-positive patients were examined for HCV core sequences. Since the recombinant c22-3 protein spans a.a. 2–120, the nucleotide sequences of the gene encoding HCV core antigen corresponding to positions 342–700 (359 nucleotides) of HCV-BK [Takamizawa et al., 1991] was determined by the dideoxy chain termination method.

The 120 deduced amino acids of the HCV core protein of the anti-core antibody-negative (N1,2), the anti-core antibody-positive patients (P1–4), and the HCV-BK are compared in Figure 1. As shown in the figure, the amino acids in 1–48 were completely conserved. As compared with HCV-BK, the amino acids of the core region in patient 1 were substituted at positions 49 and 73, and those in patient 2 were at 73 and 75. However, substitu-

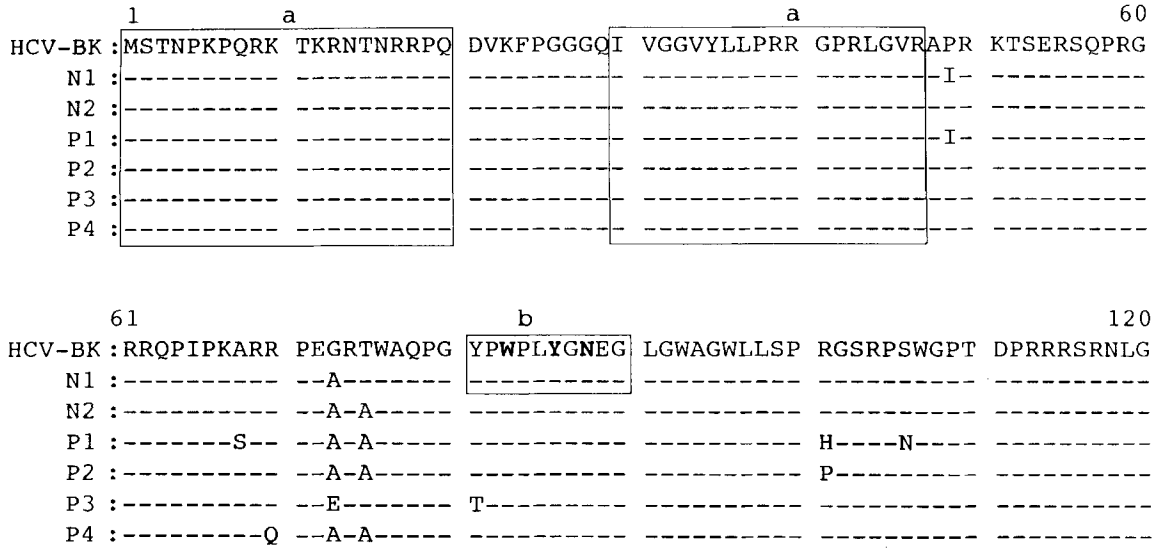

Fig. 1. Comparison of HCV core protein sequences of the patients and the HCV-BK [Takamizawa et al., 1991]. Sequences that are identical are represented by lines. [a]box indicates the B cell epitopes [Boeser et al., 1994] and [b]box indicates the HLA-DR4 binding motif [Matsushita et al., 1994]. Bold letters show the predictive anchor positions. There are no mutations in the B cell epitopes. The HLA DR4 binding motif is conserved in the sequence of N1 of patient 1 whose HLA DR was 4. N1: Which was obtained from the serum of patient 1. N2: Which was obtained from the serum of patient 2. P1–4: Core antibody-positive cases.

tion of Pro-49 of the HCV-BK core protein sequence with Ile was also seen in P1, and the conversion of Gly-73 of the HCV-BK core protein sequence to Ala was also detected in P1,2, and 4. Furthermore, substitution of Thr-75 of the HCV-BK core protein sequence with Ala was also found in P1,2, and 4. It appeared that there were no amino acid sequences specific for the HCV in the anti-core-negative patients. Additionally, core deletion variants were not found in the anti-core antibody-negative patients.

DISCUSSION

The frequency of anti-core-negative pattern only by RIBA-2 was approximately 2.0–2.3% [Bresters et al., 1993]. This may result from the sensitivity of this assay. To eliminate the possibility of merely reflecting the diagnostic limitations of assays in the two anti-core-negative patients, we used some other assays including Matrix-HCV, RIBA2, Ortho HCV Core-Ab and JCC2. Anti-core antibodies were not found in the two patients. When we studied the 93 HCV RNA-positive sera from patients with type C chronic liver disease using Matrix HCV (4 with normal ALT for at least 6 months, 64 chronic hepatitis, 7 cirrhosis, 18 hepatocellular carcinoma), anti-core antibodies were not detected in 92 out of 93 cases. Only one case (patient 1) was without anti-core antibody but positive for anti-NS3 and anti-NS4. Therefore, it is rare for HCV-infected individuals to miss only the anti-core antibody.

There are three major hydrophilic regions (a.a 2–23, 39–74, and 101–121) of the core protein which contain linear deteminants [Sallberg et al., 1992]; two of these were completely conserved in the core protein of patients N1 and N2 (see Fig. 1). Another report [Boeser et al., 1994] noted that the HCV core protein formed a three-dimensional structure exposing the two linear epitopes which were located within a.a. 1–20 and a.a. 30–47 and, in addition, these investigators described a conformational determinant within the N-terminal 69 amino acids. The two linear epitopes (a.a. 1–20 and a.a. 30–47) in these cases were also completely conserved.

Antibody production in response to protein antigens requires helper T cells. Protein antigens are processed by B cells and presented in association with class II major histocompatibility complex (MHC) molecules to antigen-specific, class II MHC restricted CD4+ helper T cells. We therefore determined the HLA DR in patient 1 and predicted the binding motif on the basis of previous work [Matsushita et al., 1994]. Patient 1 had HLA DR4 and a predictive motif for the binding of peptides to HLA DR4 present at a.a. 81–90 of HCV BK whose sequence is YPWPLYGNEG (underline indicates anchor positions). The HCV sequence of patient 1 in this region is conserved. These data indicated that the absence of anti-core in these patients was not due to the presence of previously unknown viral variants.

Yuki et al. [1994] studied the correlation between HCV replication and antibody response to c22-3, C33c, C100-3, 5-1-1, and NS5 proteins. It was shown that patients with low viremic levels and continuous normal ALT had a poor response to these proteins. Patient 1 was an asymptomatic blood donor with low viremic levels, but patient 2 had liver cirrhosis with high viremic levels. The cause of the anti-core negativity cannot therefore be fully explained by the viremic levels alone.

In the two anti-core-negative patients, HCV replicates

to a greater or lesser degree because of chronic infection and viral core proteins seem to be expressed in a similar manner to NS3 and NS4 protein. It is therefore possible that the exposure of the core antigen to the immune system of the host is in some way inhibited. The other possible explanations are that the anti-core antibodies of these patients are in some way immunocomplexed, which results in a pseudo-negativity of anti-core antibody assays, or the original infecting virus for these two patients had different gene sequences than those actually sequenced, since, in patient 1, no definite zero time point for infection could be ascertained and in patient 2 the original infection could have occurred 30 years earlier. If the original viral sequences had had mutations or deletions in the epitope regions that lead to absence of anti-core antibody in these patients, the anti-core would be positive from now on because the present infecting viral sequences in the predictive epitope regions were well-conserved. To clarify this point, follow-up of these patients with regard to core sequences and anti-core antibodies is required.

ACKNOWLEDGMENTS

This study was supported by a grant-in-aid from the Ministry of Education, Science, and Culture of Japan, and by the Research Group of Intractable Hepatitis, sponsored by the Ministry of Health and Welfare of Japan.

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